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First Record of *Philautus petilus* Stuart and Heatwole, 2004 (Amphibia: Anura: Rhacophoridae) from Vietnam and Its Phylogenetic Position

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Abstract: A small rhacophorid, *Philautus petilus* known from only the female holotype, is recorded for the first time outside of the type locality in Laos. Three specimens, containing the first known males of the species, were collected from Muong Nhe Nature Reserve in Dien Bien Province, northwestern Vietnam. The Vietnamese specimens are identified as *P. petilus* based on morphological similarities with the holotype from Laos. In addition, our molecular data verify the transfer of this species from *Philautus* to the genus *Theloderma*.

Key words: *Philautus*; *Theloderma*; New record; Phylogeny; Taxonomy; Muong Nhe Nature Reserve; Dien Bien Province

INTRODUCTION

In the checklist of amphibians and reptiles of Vietnam, Nguyen et al. (2009) reported eight species of the genus *Philautus*. However, recent phylogenetic studies suggest great changes in the generic assignment of many rhacophorid species including the species formerly nested in *Philautus*: *P. banaensis* Bourret was moved to *Kurixalus*, and *P. jinxiuensis* Hu and *P. quyeti* Nguyen, Hendrix,

Böhme, Vu, and Ziegler were transferred to *Gracixalus* by Li et al. (2008, 2009); *P. gryllus* Smith, *P. longchuanensis* Yang and Li, and *P. parvulus* (Boulenger) were placed in the genus *Raorchestes* after Biju et al. (2010); and *P. truongsongensis* Orlov and Ho was allocated to *Theloderma* by Rowley et al. (2011). Therefore, only two species of *Philautus* are currently known from Vietnam, viz. *P. abditus* Inger, Orlov and Darevsky and *P. maosonensis* Bourret (Orlov et al., 2012).

During our recent field surveys in Dien Bien Province, northwestern Vietnam, in 2012 and in 2014, three specimens of a small-sized

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rhacophorid were collected from Muong Nhe Nature Reserve. Close morphological examination of the specimens revealed them to be *Philautus petilus* Stuart and Heatwole, 2004, a species originally described from Laos and recently reassigned to the genus *Theloderma* by Stuart et al. (2013) based on morphological characteristics. Our molecular analysis also showed that this species is separated distinctly from the species of *Philautus* but clustered within the genus *Theloderma*. We herein report this species for the first time from Vietnam and confirm the transfer of this taxon from *Philautus* to *Theloderma* on the basis of molecular phylogeny.

MATERIALS AND METHODS

Sampling

Field surveys were conducted within Muong Nhe NR, Dien Bien Province, Vietnam, in

April 2012 and January 2014. Specimens were preserved in 80% ethanol after removing and fixing pieces of muscle in 95% ethanol for DNA analysis. The specimens were transferred to 70% ethanol and was subsequently deposited in the collection of the Hanoi National University of Education (HNUE), Vietnam.

Morphological analysis

Measurements were taken with a dial caliper to the nearest 0.1 mm. Terminology of morphological characters followed Stuart and Heatwole (2004). Abbreviations used are: SVL=snout-vent length; HL=head length (from tip of snout to the commissure of the jaws); HW=head width at the commissure of the jaws; SE=snout length (from tip of snout to the anterior corner of the eye); EL=eye diameter; TYD=horizontal diameter of tympanum; NS=distance from nostril to tip of

TABLE 1. Sample of *Theloderma* and outgroup species used for DNA analysis in this study together with the information on voucher, collection locality and GenBank accession numbers. Voucher abbreviations = CIB: Chengdu Institute of Biology; FMNH: Field Museum of Natural History; HNUE: Hanoi National University of Education; IABHU: Institute for Amphibian Biology, Hiroshima University; KUHE: Graduate School of Human and Environmental Studies, Kyoto University; MZB: Museum Zoologicum Bogoriense; VNMN: Vietnam National Museum of Nature.

Species	Voucher No	Genbank No.	Locality
<i>Theloderma asperum</i>	VNMN 3540	KJ802913	Vietnam, Lao Cai
<i>T. asperum</i>	VNMN J2916	KJ802914	Vietnam, Vinh Phuc
<i>T. bicolor</i>	VNMN 3536	KJ802915	Vietnam, Lao Cai
<i>T. corticale</i>	VNMN J2892	KJ802916	Vietnam, Tuyen Quang
<i>T. corticale</i>	VNMN J2932	KJ802917	Vietnam, Vinh Phuc
<i>T. gordonii</i>	VNMN PAE217	KJ802918	Vietnam, Son La
<i>T. gordonii</i>	KUHE 32447	KJ802919	Laos, Houapan
<i>T. leporosum</i>	KUHE 52581	AB847128	Malaysia, Negeri Sembilan
<i>T. licin</i>	KUHE 52599	KJ802920	Malaysia, Selangor
<i>T. rhododiscus</i>	CIB GX200807048	KJ802921	China, Guangxi
<i>T. stellatum</i>	VNMN 3686	KJ802922	Vietnam, Phu Yen
<i>T. stellatum</i>	VNMN 3687	KJ802923	Vietnam, Phu Yen
<i>Philautus aurifasciatus</i>	MZB 16395	KJ802924	Indonesia, Java, Central Java
<i>P. petilus</i>	HNUE MNA.2012.0001	KJ802925	Vietnam, Dien Bien
<i>Kurixalus eiffingeri</i>	KUHE 12910	KJ802926	Japan, Iriomote Is.
<i>Polypedates leucomystax</i>	MZB unnumbered	AB564285	Indonesia, Java, Depok
<i>Rhacophorus feae</i>	VNMN J2754	KJ802927	Vietnam, Lao Cai
<i>Nyctixalus pictus</i>	FMNH 231095	DQ283133	Malaysia, Sabah
<i>Buergeria buergeri</i>	IABHU 41011	AB127977	Japan, Hiroshima

snout; EN=distance from front of eye to nostril; UEW=maximum width of upper eyelid; IUE=minimum distance between upper eyelids; FLL=forelimb length (from the elbow to the base of outer tubercle); HAL=hand length (from the base of outer palmar tubercle to the tip of fourth toe); FL=femur length (from vent to knee); TL=tibia length; TW=tibia width; FOL=distance from the base of tarsus to the tip of fourth toe; fd1–4: width of discs of fingers I–IV, td1–5: width of discs of toes I–V. Webbing formula followed Glaw and Vences (2007).

Molecular analysis

For molecular phylogenetic analysis, we obtained tissue samples of *Philautus* and *Theloderma* from the newly collected specimens and sequences available from GenBank (trimmed to match the length of the fragment obtained here), including specimens of *Philautus aurifasciatus* (Schlegel) (type species of the genus *Philautus*) and *Theloderma leporosum* Tschudi (type species of the genus *Theloderma*). Specimens of other rhacophorid genera, *Buergeria buergeri* (Temminck and Schlegel), *Kurixalus eiffingeri* (Boettger), *Nyctixalus pictus* (Peters), *Polypedates leucomystax* (Gravenhorst), and *Rhacophorus feae* (Boulenger), were used as outgroups (Table 1).

We used the protocols of Matsui et al. (2010) for DNA extraction, amplification, and sequencing. Fragments containing 12S rRNA, tRNA^{Val}, and 16S rRNA, approximately 2250 bp long, were amplified and sequenced using six pairs of primers (Kuraishi et al., 2011). Sequences were aligned by ClustalX (Thompson et al., 1997) and manually checked using the original chromatograph data in the program BioEdit (Hall, 1999). Phylogenetic trees were constructed using Maximum Likelihood (ML) and Bayesian Inference (BI). We used Treefinder ver. 1.5 Oct. 2011 (Jobb, 2011) for ML and MrBayes ver 3.1.2 (Ronquist and Huelsenbeck, 2003) for BI. Tree nodes with bootstrap values of 70% or greater were considered as sufficiently resolved (Hillis and Bull, 1993). In the BI analysis, nodes with a

BPP of 95% or greater were considered significant (Leache and Reeder, 2002). Uncorrected p-distances for 16S rRNA were calculated by using MEGA ver. 5.2.

RESULTS

Details of the three Vietnamese specimens examined are as follows: HNUE MNA.2012.0001 (adult male) collected on 14 April 2012, and HNUE MNA.2014.0374 (adult female) and HNUE MNA.2014.0375 (adult male) collected on 21 January 2014, all by Dzung Trung Le, from near the Phy Thy stream (22°19'54"N 102°20'55"E, 620 m asl) within Muong Nhe Nature Reserve, Leng Su Sin Commune, Muong Nhe District, Dien Bien Province, Vietnam.

Description of the specimens from Vietnam

Habitus moderately slender, body dorsoventrally compressed (SVL 31.5–32.4 mm in two males; 30.3 mm in a female); head longer than wide (HL 9.8–10.1 mm, HW 8.2 mm, HL/SVL 0.31, HW/SVL 0.25–0.26 in males and HL 10.1 mm, HW 8.1 mm, HL/SVL 0.33, HW/SVL 0.27 in the female); snout slightly pointed anteriorly (SE 3.8–4.0 mm, SE/SVL 0.12–0.13 in males and SE 3.6 mm, SE/SVL 0.12 in the female); eye large, shorter than snout length (EL 3.5 mm, EL/HL 0.35–0.36, EL/SE 0.88–0.92 in males and EL 3.1 mm, EL/HL 0.31, EL/SE 0.86 in the female), pupil round; tympanum round, small, clearly visible (TYD 2.2–2.3 mm, TYD/EL 0.63–0.66 in males and TYD 2.2 mm, TYD/EL 0.71 in the female); supratympanic fold distinct; nostril in lateral direction, much closer to tip of snout than to eye (NS 1.2–1.3 mm, EN 3.9–4.1 mm, NS/EN 0.31–0.32 in males and NS 1.3 mm, EN 4.2 mm, NS/EN 0.31 in the female); loreal region slightly concave, oblique; interorbital distance narrower than upper eyelid (IUE 2.4 mm for all specimens, IUE/UEW 1.5–1.6 in males and 1.5 in the female); vomerine teeth very small, in oblique rows closer to choanae than to each other; tongue deeply notched posteriorly.

Forelimb slender and short (FLL/SVL 0.17–0.19 in males and 0.17 in the female); relative lengths of fingers: $I < II < IV < III$; tips of fingers with round discs, with circummarginal grooves; disc of finger III smaller than tympanum (fd3/TYD 0.7–0.89 in males and 0.65 in the female); fingers free of webbing; fingers III and IV with large middle subarticular tubercle, inner palmar tubercle small, outer palmar tubercle distinct; fingers I and II with large palmar tubercle at base.

Thigh long; tibia approximately five times longer than wide; relative toe length $I < II < III < V < IV$; tips of toes expanded into round discs, with circum-marginal grooves, slightly smaller than finger discs; subarticular tubercles on toes I–V: 1, 2, 2, 4, 3; webbing formula $Ie(1/2)(1)iIe(1/2)(1)iIIe(1/2)(1)iIVe(1)(0)iV$; inner metatarsal tubercle elongated, outer metatarsal tubercle very small, almost indis-

cernible.

Skin on dorsal and ventral surfaces smooth, except for distinct, white asperities on head, posterior part of back, dorsal surfaces of forelimb, thigh, tibia, fingers and toes, and anterior half of sides; dermal fringes, row of enlarged tubercles, or accessory flaps of skin absent on outer margins of limbs.

Coloration in life, head and body dorsally light brown with dark brown reticulations and black spots of irregular shape, larger and more distinct in posterior part of dorsum; dorso-lateral zone of head and body light brown; lateral head and tympanum dark brown, darker from behind tympanum to groin; a black stripe present below edge of canthus extending from tip of snout to anterior corner of eye and from posterior corner of eye along supratympanic fold to flank, edged in white near level of mid-body; axillar region white; dorsal surface of limbs dark brown with white asperities; thigh and tibia with some black marbling; venter cream, chin with some dark spots, underside of limbs pigmented; pupil black; iris bicolored, upper part reddish brown, lower part grey (Fig. 1).



FIG. 1. A male *Thelodermatops petilum* (HNUE MNA.2012.0001) from Dien Bien Province, Vietnam: A) Dorsal view and B) ventral view.

Natural history

The first male (HNUE MNA.2012.0001) was found at 20:30 h on the tree, ca. 0.7 m above the ground. The second male (HNUE MNA.2014.0375) and the female (HNUE MNA.2014.0374) were found in the water in a bamboo hole. Surrounding habitat is evergreen mixed forest of hardwoods and bamboos at an elevation of 623 m asl. The advertisement call and larvae of this species are unknown.

Morphological identification

The specimens from Vietnam agree well with diagnosis of the species although they differ from the female holotype from Laos by having a longer head (ratio of HL/SVL 0.31 in males and 0.33 in the female vs. 0.27 in the holotype). In contrast, the Vietnamese specimens have smaller ratios of tympanum to eye diameter (TYD/EL 0.63–0.66 in males and

0.71 in the female vs. 0.80 in the holotype) (see Stuart and Heatwole, 2004). However, we suppose that these differences might be within the range of individual variation. Morphological characteristics of the Vietnamese specimens (e.g., the presence of dorsal dermal asperities and distinct tympanum, and the absence of finger webbing) also supported the removal of this species from *Philautus* to *Theloderma* as suggested by Stuart et al. (2013).

Phylogenetic position of *Philautus petilus*

In order to identify the phylogenetic position of the species, we used molecular evidence to provide independent support of its generic assignment from morphology. The combined matrix contained 642 aligned characters, of which 340 were variable and 273

were parsimony-informative for ingroup. The best model selected by Kakusan for both analyses was GTR with gamma (0.341 for ML and 0.352 for BI). The ML and BI analyses produced essentially identical topology, therefore, only ML tree is shown in Fig. 2. The likelihood values (-lnL) for ML and BI tree were 4889 and 4913, respectively. In our analyses, the specimen from Dien Bien Province was embedded within the clade II of the genus *Theloderma*, together with *T. asperum* (Boulenger) and *T. licin* McLeod and Norhayati. This clade is clearly separated from the clade I (*T. bicolor* [Bourret], *T. corticale* [Boulenger], *T. gordonii* Taylor, *T. rhododiscus* [Liu and Hu], and the type species of the genus, *T. leporosum* Tschudi, from Malaysia) and the clade III (*T. stellatum* Taylor) (see Fig. 2). The Vietnamese

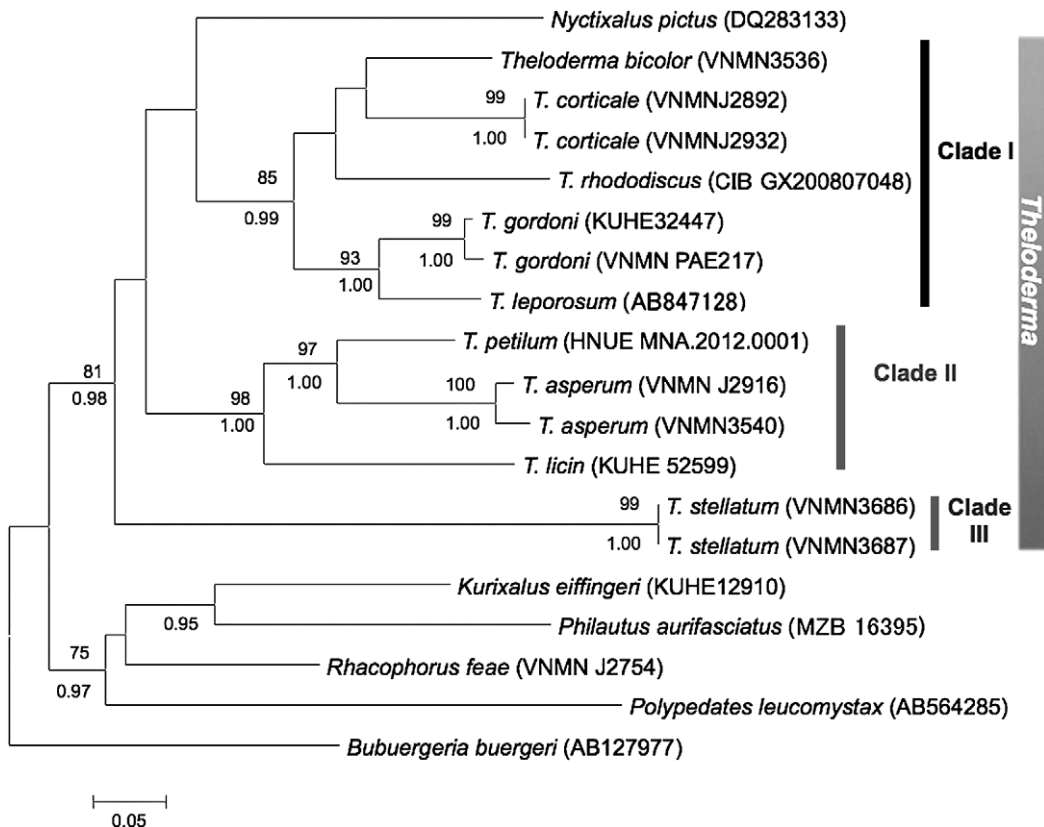


FIG. 2. Maximum-likelihood (ML) tree of *Theloderma petilus* and related taxa based on the partial 16S rRNA mitochondrial gene. Numbers above and below branches are ML bootstrap values (values ≥ 70 shown) and Bayesian posterior probabilities (values ≥ 0.95 shown).

TABLE 2. Uncorrected p-distances (in %) for short fragment of 16S rRNA among species in the genus *Theloderma* and outgroup species.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 <i>Theloderma asperum</i> (VNMN 3540)	—																	
2 <i>T. asperum</i> (VNMN 12916)	2.9	—																
3 <i>T. bicolor</i> (VNMN 3536)	18.5	19.2	—															
4 <i>T. corticale</i> (VNMN 12892)	18.5	18.5	10.1	—														
5 <i>T. corticale</i> (VNMN 12932)	18.5	18.5	10.1	0.0	—													
6 <i>T. gordonii</i> (VNMN PAE217)	17.4	17.6	12.1	13.5	13.5	—												
7 <i>T. gordonii</i> (KUHE 32447)	17.6	17.8	12.8	13.7	13.7	1.1	—											
8 <i>T. leporosum</i> (AB847128)	19.2	19.8	12.4	13.5	13.5	8.3	7.6	—										
9 <i>T. licin</i> (KUHE 52599)	14.9	15.3	17.8	19.2	19.2	17.3	17.4	17.8	—									
10 <i>T. rhododiscus</i> (CIB GX200807048)	18.2	18.3	13.5	13.1	13.1	14.0	14.0	13.7	18.5	—								
11 <i>T. stellatum</i> (VNMN 3686)	22.7	23.0	18.2	20.0	20.0	20.3	20.9	20.5	21.6	21.8	—							
12 <i>T. stellatum</i> (VNMN 3687)	22.7	23.0	18.2	20.0	20.0	20.3	20.9	20.5	21.6	21.8	0.0	—						
13 <i>Philautus aurifasciatus</i> (MZB 16395)	21.0	21.4	19.4	19.6	19.6	19.2	19.1	17.8	19.1	22.3	21.8	21.8	—					
14 <i>T. petilus</i> (HNUE MNA.2012.0001)	11.2	11.7	15.8	16.2	16.2	14.7	15.1	16.4	15.3	18.3	22.7	22.7	19.8	—				
15 <i>Kurixarus eiffingeri</i> (KUHE 12910)	21.4	20.3	17.8	18.7	18.7	17.6	18.0	17.8	20.1	20.7	22.1	22.1	16.9	18.9	—			
16 <i>Polypedates leucomystax</i> (AB564285)	21.8	21.4	20.3	20.7	20.7	20.5	21.4	21.4	22.5	20.9	25.5	25.5	21.9	21.9	18.7	—		
17 <i>Rhacophorus feae</i> (VNMN 12754)	18.7	18.5	18.5	18.2	18.2	15.5	15.6	16.5	18.2	17.8	20.1	20.1	16.4	18.2	13.7	20.0	—	
18 <i>Nyctialus pictus</i> (DQ283133)	18.3	19.2	16.0	16.7	16.7	16.0	16.0	18.5	15.8	16.4	21.2	21.2	19.2	18.7	17.3	20.9	16.9	—
19 <i>Buergeria buergeri</i> (AB127977)	19.1	19.8	18.5	18.7	18.7	17.8	17.8	18.0	19.6	19.2	22.3	22.3	19.4	18.7	19.1	20.3	16.0	21.4

specimen of *Philautus petilus* is significantly divergent from others within the Clade II with the minimum p-distance of approximately 11.2% in the mitochondrial fragment of 16S rRNA (see Table 2). Based on these phylogenetic results, we confirm the placement of *Philautus petilus* in the genus *Theloderma*.

DISCUSSION

As mentioned above, recent systematic studies have made great changes in the generic allocation of many rhacophorid species, including the change in the contents of the genus *Theloderma*. These changes were made mostly based on results of molecular phylogenetic analyses (e.g., Frost et al., 2006; Li et al., 2009; Yu et al., 2009), and generic diagnoses based on morphology are becoming more and more obscure within Rhacophoridae. This is also the case with *Theloderma* as shown above and morphological synapomorphies to delimit the genus are still to be investigated. Although most authors agree that the genera *Theloderma* and *Nyctixalus* are monophyletic (Pyron and Wiens, 2011; Rowley et al., 2011), some recent phylogenetic work threw doubt on the monophyly of *Theloderma*. Our result, with three groups in this genus concurred this, although *Philautus petilus* was clearly nested within one group of *Theloderma*.

Since the original description of Stuart and Heatwole (2004), only a single female specimen of *Theloderma petilum* has been known from the type locality, Phou Den Din National Biodiversity Conservation Area in northern Laos. The specimens from Muong Nhe Nature Reserve are the first record in Vietnam and also provided knowledge on males of this species. It is noted that Muong Nhe Nature Reserve of Vietnam is contiguous with the Phou Den Din National Biodiversity Conservation Area of Laos and the newly recorded locality is approximately 30 km eastward from the type locality of the species (Fig. 3).

Vietnam is the type locality of 10 species (or 46% of the species number) of the genus

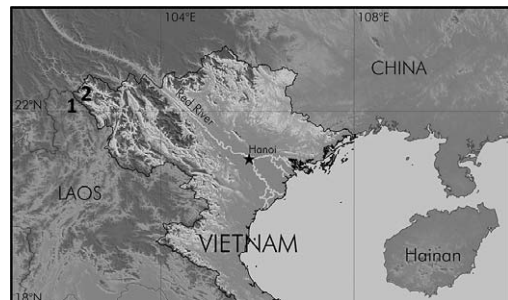


FIG. 3. Distribution of *Theloderma petilum*: 1) Phou Dendin National Biodiversity Conservation Area, Phongsaly, Laos (type locality) and 2) Muong Nhe Nature Reserve, Dien Bien Province, Vietnam.

Theloderma. Since 2009, five species, *T. bambusicolum* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Nguyen and Geissler; *T. chuyangsinensis* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Nguyen and Geissler; *T. lateriticum* Bain, Nguyen and Doan; *T. nebulosum* Rowley, Le, Hoang, Dau and Cao; and *T. palliatum* Rowley, Le, Hoang, Dau and Cao, have been described from the country (Bain et al., 2009; Rowley et al., 2011; Orlov et al., 2012), and our finding of *T. petilum* brings the species number of *Theloderma* to 15 in Vietnam (Nguyen et al., 2009; Orlov et al., 2012). Vietnam has been considered as a hotspot of new species discovery in Asia (Nguyen, 2006; Ziegler and Nguyen, 2010), but new recordings of species known from adjacent regions like the present case also will further augment this country's rich amphibian fauna.

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